

TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. 371

101195-48

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR

09/831083

INTERNATIONAL APPLICATION NO.

PCT/DE99/03432

INTERNATIONAL FILING DATE

27 Oct. 1999 (27.10.99)

PRIORITY DATE CLAIMED

4 November 1998 (04.11.98)

TITLE OF INVENTION

New Expression Cassette for Expression of Arbitrary Genes in Plant Seeds

APPLICANT(S) FOR DO/EO/US

Ute Heim; and Hans Weber

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☐ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
 - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☒ has been transmitted by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☐ A copy of the International Search Report (PCT/ISA/210).
8. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ have been transmitted by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☐ have not been made and will not be made.
9. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
10. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
11. ☐ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☒ Certificate of Mailing by Express Mail
20. ☐ Other items or information:

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR 1.53) 09/831083		INTERNATIONAL APPLICATION NO. PCT/DE99/03432		ATTORNEY'S DOCKET NUMBER 101195-48	
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21. The following fees are submitted: BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) : <input type="checkbox"/> Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO \$1,000.00 <input checked="" type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO \$860.00 <input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$710.00 <input type="checkbox"/> International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$690.00 <input type="checkbox"/> International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) \$100.00 <div style="text-align: right;">ENTER APPROPRIATE BASIC FEE AMOUNT =</div>				CALCULATIONS PTO USE ONLY	
				<div style="border: 1px solid black; width: 100px; height: 100px; margin: 0 auto;"></div>	
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input checked="" type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).				\$860.00	
				\$130.00	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	- 20 =	0	x \$18.00	\$0.00	
Independent claims	- 3 =	0	x \$80.00	\$0.00	
Multiple Dependent Claims (check if applicable). <input type="checkbox"/>				\$0.00	
TOTAL OF ABOVE CALCULATIONS =				\$990.00	
Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28) (check if applicable). <input checked="" type="checkbox"/>				\$495.00	
SUBTOTAL =				\$495.00	
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492 (f)).				\$0.00	
TOTAL NATIONAL FEE =				\$495.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable). <input type="checkbox"/>				\$0.00	
TOTAL FEES ENCLOSED =				\$495.00	
				Amount to be: refunded \$	
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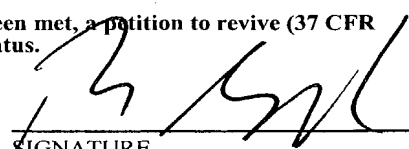
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☒ Please charge my Deposit Account No. **14-1263** in the amount of **\$495.00** to cover the above fees.
 A duplicate copy of this sheet is enclosed.
☒ The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. **14-1263** A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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PATENT TRADEMARK OFFICE



SIGNATURE

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NAME

33-531

REGISTRATION NUMBER

May 3, 2001

DATE

09/831083

JC08 Rec'd PCT/PTO 03 MAY 2007

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Atty's Docket No. 101195-48

APPLICANT : Ute Heim et al.
FILED : Concurrently Herewith
FOR : New Expression Cassette for Expression of
Arbitrary Genes in Plant Seed

PRELIMINARY AMENDMENT

Hon. Assistant Commissioner of Patents
Box PCT
Washington, D.C. 20231

Sir:

Prior to examination, please amend the application as
follows:

IN THE SPECIFICATION

Page 1, line 3, please delete "Description" and insert
--Background of the Invention--;

Page 2, after line 21 (using printed line numbers), please
insert --Summary of the Invention--;

after line 32 (using printed line numbers), please
insert --Brief Description of the Drawings

Fig. 1 - the sequence of SBP promoter;

Fig. 2a - Northern blot of Vicia faba against VFSBP20 probe;

- Fig. 2b - cross-section of ripe Transgenic (SBPRGUS) tobacco seed;
- Fig. 2c - β -glucuronidase content in transgenic pSBPRGUS tobacco line;
- Fig. 3 - restriction maps of clone pSBPR7 and pSBPR15;
- Fig. 4 - a graft of plasmid pGPTV-Bar;
- Fig. 5 - a graft of the 3' untranslated area of promoter region with the polyadenylation signals of the octopine synthase gene;
- Fig. 6 - a graft of the smoothed Asp719/SphI fragment ligated with the binary vector pGPTV-Bar from plasmid pSBRXYNZ;
- and
- Fig. 7 - Western Blot of protein extract from ripe seed with Xylanase Z directed antibodies.

Detailed Description of the Preferred Embodiments--;

Page 4, line 12 (using printed line numbers), please insert --blot-- after "Northern".

A clean copy of this addition and amended page 4 is attached.

IN THE CLAIMS

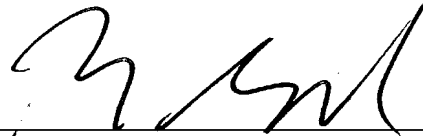
Please amend the claims in accordance with the attached

marked-up pages. A clean copy of the amended claims is also enclosed.

REMARKS

The above amendments were made to place the application into proper United States Patent Format.

Respectfully Submitted,



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Clean copy of addition to specification on page 2, after line 32

Brief Description of the Drawings

- Fig. 1 - the sequence of SBP promoter;
- Fig. 2a - Northern blot of Vicia faba against VFSBP20 probe;
- Fig. 2b - cross-section of ripe Transgenic (SBPRGUS) tobacco seed;
- Fig. 2c - β -glucuronidase content in transgenic pSBPRGUS tobacco line;
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- and
- Fig. 7 - Western Blot of protein extract from ripe seed with Xylanase Z directed antibodies.

Detailed Description of the Preferred Embodiments

Clean copy of amended page 4 of the specification

The nucleotide sequence of the expression cassette contains transcriptionally regulatory areas, guaranteeing a strong specific expression of an arbitrary gene into the seed of plants. The Northern blot (Fig. 2a) shows the high seed-specific expression in the various tissues of *Vicia faba*. The GUS data in Figs. 2b and 2c show on the one hand the distribution of the β -glucuronidase in the sections through ripe tobacco seeds and, on the other, the accumulation of the β -glucuronidase in the transgenic tobacco seeds as a function of development.

Marked-up copy of amended page 4 of the specification

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7/PRTS

09/831083

JC08 Rec'd PCT/PTO 03 MAY 2001

New expression cassette for expression of arbitrary genes in plant seeds

Description

5

The invention in question relates to an expression cassette for expression of arbitrary genes in plant seeds and the plasmids containing the expression cassette. The invention also includes the production of transgenic plant cells containing this expression cassette as well as the use of the plasmids in this expression cassette for production of transgenic plants. Fields of application of the invention are biotechnology, pharmacy and plant production.

15 For a long time now, there have been methods making it possible to integrate relevant genes into the genome of higher plants. The objective of this work is the production of plants with new properties, for example to increase agricultural production, to optimise manufacture of foodstuffs and
20 to produce specific pharmaceuticals and other interesting ingredients. One prerequisite for the expression of the transferred genes in this context is that they possess plant-specific promoter sequences. For this purpose, so-called constitutive promoters such as the promoter of the nopaline synthase gene /1/, the TR double promoter /2/ or the promoter of
25 the 35S transcript of the cauliflower mosaic virus /3/ are used. One disadvantage of these promoters is that they are active in almost all the tissues of the manipulated plants. In this way, a controlled and purposeful expression of the
30 foreign genes in the plants is not possible. It is better to use promoters which function tissue-specifically and independently of development. Genes with the matching promoters, which are only active in anthera, ovaries, blooms, leaves, deciduous leaves, stems, roots or seeds, have been isolated
35 /4/. But they differ greatly in the strength and specificity of the expression and only have a limited use. For the use of the seeds as a source of nutrition and for production of in-

gredients, it is above all the seed-specific promoters which are of great interest. With the years of research into the genes of the seed-storage proteins, some more or less specific promoters with differing strengths, for example that of phaseolin /5/ or legumin and USP /6/ are available. As these storage proteins are synthesised by gene families, fusions of such promoters with foreign genes are in competition with the endogenous numerous genes of the corresponding gene family. For this reason, it is more favourable to use promoters from unique, strongly and specifically expressing genes. For co- and multiple transformations, the use of differing regulatory sequences is suitable, in order to make better use of the development of the seed in time, to synthesise identical or differing gene products in parallel and to avoid co-suppression.

Although a number of expression cassettes for expression of arbitrary genes in plant seeds are already known, the expression rates in plant seeds achieved have not been optimal up to now for the substantiation of a plant biotechnological production of the required materials.

The invention therefore has the objective of placing the seed-specific expression in transgenic plants on a basis suitable for a production of materials. It is based on the task of constructing an expression cassette with which a stable expression with a high expression rate of genes of the materials to be produced can be achieved in plant seeds.

The objective of the invention is achieved with the expression cassette described in claim 1, with sub-claims 2-7 being preferred variants.

The expression cassette according to the invention contains the following essential component parts:

- the promoter of the gene of the sucrose binding protein (SBP)like protein

- if applicable, the DNA sequence of a signal peptide, preferably the SBP signal peptide
- a gene to be expressed
- 3' termination sequences

5 The invention relates above all to a regulatory DNA sequence occurring uniquely in the genome, which mediates a strong expression of an arbitrary heterologous gene primarily in the cotyledons and in the endosperm dependency on seed development.

10 The most important component part of the cassette is the SBP promoter, the sequence of which is shown in Figure 1. Compared with analog promoters in this field, this promoter has the benefit of great strength and seed-specificity. Its use for the expression of foreign genes, even without the DNA sequence of a signal peptide, is also part of the scope of the
15 invention.

Together with the transcriptionally regulatory sequences, the expression cassette also, if need be, contains a signal peptide, which enables the transport of the required gene product into the protein bodies, thus preventing decomposition of the gene products to a great extent. The optional use of the authentic signal peptide enables the transport of the synthesised foreign proteins to and storage in the protein bodies.

25 The genes to be expressed can be integrated either as transcription or as translation fusions, they can be varied to a great extent, for example genes can be used for the production of enzymes (e.g. amylase, xylanase), pharmaceutical products or for the over-expression of proteins with a high
30 share of essential amino-acids (e.g. 2S globulin of the brazil nut rich in methionine) or of other proteins influencing the properties of the seeds. Further possibilities can be found in the reduction or elimination of gene products
35 through the integration of genes in an anti-sense orientation. By inserting regulatory genes under the control of this seed-specific promoter, metabolic processes in the seeds can

also be influenced. The cassette can also be used in order to express the SBP gene inherent to the promoter from field beans into other species. The use of other terminators, for example the termination sequence of the gene to be expressed, is a further possibility of optimal use of the cassette. As a concrete example, the gene of β -glucuronidase (GUS) was used to show the specificity of the promoter (Fig. 2b, c).

The nucleotide sequence of the expression cassette contains transcriptionally regulatory areas, guaranteeing a strong specific expression of an arbitrary gene into the seed of plants. The Northern (Fig. 2a) shows the high seed-specific expression in the various tissues of *Vicia faba*. The GUS data in Figs. 2b and 2c show on the one hand the distribution of the β -glucuronidase in the sections through ripe tobacco seeds and, on the other, the accumulation of the β -glucuronidase in the transgenic tobacco seeds as a function of development.

The plasmids containing the expression cassette, preferably the plasmids pSBPROCS and pPTVSBPRGUS, are also to be placed under protection.

The scope of the invention also includes the use of the expression cassette according to claims 12-16, which is done by transformation into bacteria strains and subsequent transfer of the resulting recombinant clones into preferably dicotyl plants. The plants expressing the required gene product in the seed are selected and bred as genetically stable lines. After harvesting, the required gene products are extracted from the transgenic seeds in a way basically already known.

This invention is also interesting for applications in which the required gene product is expressed under the control of various promoters, in order to increase the total of the expression rates, in order to make better use of the development period of the seeds and to avoid effects by co-suppression. This expression cassette is also suited for co- and

multiple transformations with the objective of expressing various gene products. A variety of new expression cassettes is needed for these strategies in order to be able to select the correct ones.

5

The entire method for the alteration of a plant cell is portrayed in an example (pSBPOCS).

The invention is to be explained in more detail below with
10 examples of embodiments.

Methods

1. Cloning method

15 For cloning, the vectors pUC18 /7/, pBK-CMV (Stratagene) and pOCS1 (Plant Genetic Systems, Gent, Belgium) and for plant transformation the vectors BIN19 /8/, and, after deletion of the GUS gene, pGPTV-BAR /9/ were used.

20 2. Bacteria strains

For the transformation to E. coli, strain DH5 α /10/ was used. The binary plasmids were inserted into the agro-bacteria strain EHA105 /11/ by conjugation.

25 3. Plant transformation

The transformation of *Nicotiana tabacum* was done by the leafdisk method /12/ and the transformation of *Vicia narbonensis* with the help of the method described by Pickardt in 1991 /13/ by agrobacterium mediated gene transfer.

30

4. Analysis of genomic DNA from transgenic plants

The genomic DNA of the transgenic tobacco and *V. narbonensis* plants was isolated with the help of the DNA isolation kit of the firm of Macherey & Nagel. In a first step, the transgenic
35 lines were identified via PCR with gene-specific primers. The integration of foreign DNA was examined by means of "Southern

blot" analyses of 20 μ g of DNA following suitable restriction digestion.

5. β -glucuronidase activity test (GUS assay)

- 5 The reporter gene β -glucuronidase is a bacterial enzyme accessible to both quantitative /14/ and also histo-chemical activity assays. Tissue samples were incubated over night at 37°C in 1 mM X-Gluc, 50mM Na phosphate (pH 7.0) and 0.1% Tween 20. For sections, the tissues were fixed, embedded in
10 paraffin and cut to a section thickness of 15 - 30 μ m on a microtome.

Examples of embodiments

- 15 The invention, which contains the production of a new, seed-specific expression cassette as well as the plasmids and transgenic plants derived from them, is explained below - partly with the help of the figures - using an example of an embodiment.

20

1.) Cloning and structure analysis of an SBP seed protein gene from *Vicia faba*

- Primers (5'-GAAGACCCTGAGCTCGTAACTTGCAA-ACAC- 3' and 5'-AGTACTCATAGATCTCTGGGTGATGTTGGT-3') were derived from the sequence of a cDNA clone which codes for the sucrose binding protein of the soybean /15/. The gene-specific probe was then amplified, cloned and sequenced by means of RT - PCR on mRNA, isolated from immature cotyledons of *V. faba*. The PCR product was identified as the gene fragment homologous to the sucrose
25 binding protein and was used as a probe for the isolation of the complete cDNA from a cotyledon-specific λ Zap Express cDNA Bank of *V. faba* L. var. minor. One of the isolated clones (VfSBP20), which has a homology of 68% on the nucleotide level, codes for the complete SBP-homologous gene
30 from the field bean. But it differs from the gene isolated from the soybean in both the expression (Fig. 2a) and also in the function (no sucrose binding).
35

2) Isolation of the regulatory sequences by means of PCR

The regulatory sequences were isolated with the help of the "Universal GenomeWalkerTMKit" of the firm of CLONTECH and the

5 gene-specific primers PSBP1, position 159 (5'-AATCCTCA-
CACTTCTCCATGCATATCCGTTTGTCC-3'), PSBP2, position 118 (5'-
GCCCTGCAGAT-CGCATTTGTCTTTGCA-3') and PSBP3, position 85 (5'-
CTGGGTCCTTTTCTTTTCTGG- C-3'). Following prior digestion of
10 the genomic DNA of *V.faba* with ScaI (a) and StuI (b) and
ligation of the adapters, a two-step PCR was done in accor-
dance with the description of the kit with the following pa-
rameters: 7 cycles of 94°C, 2s, 72°C, 3 min and 32 cycles of
94°C, 2s, 67°C, 3 min and 4 min 67°C. The PCR preparations
15 were diluted 1:50 and 1µl of each were amplified in a second
PCR (5 cycles of 94°C, 2s, 72°C, 3 min and 20 cycles of 94°C,
2s, 67°C and 4 min at 67°C. In the Agarosegel, bands of 1.7 kb
from (a) and 1.9 kb from (b) were verified via a Southern
blot. These bands were then cloned into the pUC18 and se-
quenced. The clones SBPR7 and SBPR15 were then identified by
20 a sequence comparison as the promoters matching gene VfSBP20.
They represent allelic variants of gene VfSBP20, with both
clones having 100% sequence identity with clone VfSBP20 in
the corresponding area. On the 5' side of the ATG of the SBP
gene, 1539 bp were isolated with clone SBPR7 and 1750 bp with
25 clone SBPR15. They differ by 23 base pair substitutions and
two insertions. The restriction maps of clone pSBPR7 and
pSBPR15 are shown in Fig. 3, the sequence of clone pSBPR15 in
Fig. 1.

30 3a) Proof of the seed-specific expression in tobacco

With the help of the reporter gene of β -glucuronidase, the
seed-specific expression of the isolated regulatory sequences
SBPR7 and SBPR15 was to be tested. For this, the binary plas-
mid pBI101 /14/, which contains the promoter-free glucuroni-
35 dase gene behind a poly-linker, was cut with SmaI and dephos-
phorylated. The promoters were isolated from the plasmids
pSBPR7 and pSBPR15 respectively by means of an SalI/NcoI

digestion and the ends smoothed. The fragments were then cloned into the SmaI site of binary plasmids pBI101 in front of the reporter gene, with plasmids pBISBPR7GUS and pBISBPR15GUS resulting. These plasmids were then transferred to the agro-bacteria strain EHA105 and the chimerical agro-bacteria containing SBP promoter/glucuronidase gene were used for the transformation of tobacco. The results are shown in Figures 2b and 2c. The analysis of the transgenic tobacco seeds shows a strong blue coloration and thus a strong activity of the glucuronidase in the endosperm and in the cotyledons of the tobacco seeds, also according to the seed development. No glucuronidase activity was detected in other tissues. The two slightly different nucleotide sequences SBPR7 and SBPR15 also do not differ in their expression behaviour. These data show that the isolated regulatory sequences fused with the β -glucuronidase gene result in a strong and strictly seed-specific expression in the tobacco.

3b) Proof of the seed-specific expression in peas

In order to show that a seed-specific expression is also to be expected in legumes, the SalI/NcoI fragment of plasmid pSBPR15 was cloned into the SalI/NcoI cut plasmid pGUS1 (Plant Genetic Systems, Gent). From the resulting plasmid pSBPGUS, the fusion of the SBPR15 promoter/GUS/ocs-terminator was cut out with SalI/SmaI, smoothed and ligated into the binary plasmid pGPTV-Bar, EcoRI/SmaI cut (Fig. 4). pGPTV-Bar/9/ is a binary plasmid mediating phosphinothricin resistance which is successfully used for the transformation of peas. This plasmid has been called pPTVSBPRGUS (Fig. 4). The embryos of the transgenic pea lines generated with this plasmid show a strong blue coloration after a histo-chemical analysis.

3c) Proof of the transient expression in embryos of Vicia

faba, Vicia narbonensis, Pisum sativum and Brassica napus
With plasmid pSBPGUS, isolated embryos of Vicia faba, Vicia narbonensis, Pisum sativum and Brassica napus were shot by

means of the Biolistics PDS-1000/He Particle Delivery System under the following conditions. The coating preparation comprised 50 μ l of gold (Hereaus, 0.6-3 μ m, 50 mg/ml), 10 μ l of Qiagen-cleaned plasmid-DNA (1 μ g/ μ l), 50 μ l of 2.5M CaCl₂ and 10 μ l of 0.1M spermidine. At 1800 Psi and a vacuum of 27 inch Hg, the embryos lying on an agar panel were then shot and subsequently cultivated in MS-2% sucrose liquid medium for 2 days. There was then a reaction over night at 37°C with X-Gluc (1mM) in 50mM Na phosphate (pH 7.0) and 0.1% Tween 20. Unlike the negative control (promoter-free pGUS1), a number of blue dots were registered in the above mentioned embryos, showing that the SBP promoter functions in the seeds.

4.) Production of the expression cassette for over-expression of heterologous genes in the seed

In order to make the regulatory sequences available for the over-expression of foreign genes, the SalI fragment of the longer clone SBPR15 was isolated and smoothed and cloned into the SmaI location of plasmid pOCS1 (Plant Genetic Systems, Gent, Belgium). This cassette thus contains the promoter region, the complete 5' untranslated region, the complete signal peptide, the first five triplets of the ripe protein (Fig. 1) and the 3' untranslated area with the polyadenylation signals of the octopine synthase gene (Fig. 5). The NcoI location can be used for transcription fusions with foreign genes, the BamHI location for translation fusions. After the insertion of the foreign gene, the sequence containing the promoter, regulatory sequences, the foreign gene and the 3' termination sequences is cut out with restriction enzymes and cloned into a binary vector with the herbicide resistance suitable for the plant transformation.

As an example of this, the BamHI fragment of the gene of XylanaseZ of Clostridium thermocellum was cloned into the BamHI location of plasmid pSBPOCS as a translation fusion. From the resulting plasmid pSBPRXYNZ (Fig. 6), the smoothed Asp718/SphI fragment was ligated with the binary vector

pGPTV-Bar, which was cut with the enzymes EcoRI/SmaI and smoothed. After transformation into the agro-bacteria strain EHA105, *N. Tabacum* was transformed. The strong expression of the Xylanase Z was shown in the ripe transgenic seeds in a
 5 Western blot (Fig. 7).

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and Hitz,W.D. (1992) The Plant Cell, 4, 1561-1574.

Patent claims

1. New expression cassette for expression of arbitrary genes in plant seeds, comprising
 - the promoter of the gene of the seed protein similar to the sucrose binding protein (SBP)
 - if applicable, the DNA sequence of a signal peptide, preferably the SBP signal peptide
 - a gene to be expressed
 - 3' termination sequences
2. Expression cassette according to claim 1, wherein it contains the SBPR promoter with the sequence corresponding to Fig. 1 without a DNA sequence of a signal peptide.
3. Expression cassette according to claims 1 and 2, wherein a further DNA sequence is downstream to the DNA region provided with a transcriptionally regulatory sequence for a strong seed-specific gene expression, the latter region containing the information for the formation and quantitative distribution of endogenous products or the expression of heterologous products in culture crops.
4. Expression cassette according to claims 1 to 3, wherein arbitrary foreign genes are integrated either as transcription or as translation fusions.
5. Expression cassette according to claims 1 to 4, wherein the signal peptide of the SBP seed protein gene is used as a signal peptide.
6. Expression cassette according to claims 1 to 5, wherein the gene of the sucrose binding protein like gene is used as the gene to be expressed.

7. Expression cassette according to claims 1-6, wherein it is also used for co- and multiple transformations.

5 8. Plasmids containing an expression cassette according to claims 1-5.

10 9. Plasmid pSBPROCS, comprising a DNA sequence about 5.3 kB in size, in which a SalI promoter fragment of the regulatory starter area about 1.9 kb in size including the signal peptide and 5 triplets of the SBP-homologous gene of Vicia-faba, restriction sites for cloning in foreign genes and the transcription terminator of the octopine synthase gene are contained.

15

10. Plasmid pPTVSBPRGUS, comprising a DNA sequence about 14.9 kb in size, in which a phosphinothricin resistance gene about 1 kb in size, a SalI/NcoI promoter fragment of the regulatory starter area of the SBP-like gene of Vicia faba about 1.8 kb in size, the coding region of the β -glucuronidase about 1 kb in size and the transcription terminator of the octopine synthase gene are contained.

20

11. Method for the insertion of an expression cassette with a DNA sequence for strong seed-specific gene expression into a plant cell, comprising the following steps:

25

- a) isolation of clone VfSBP20, wherein the gene coding for the SBP seed protein occurring in the plant seed is selected from a cDNA Bank of cotyledons of Vicia faba,
- 30 b) isolation of clone pSBPR15, wherein the DNA sequence contained therein comprises the regulatory starter region of the SBP seed protein gene of Vicia faba and a sequence from a related legume hybridising with the DNA sequence of the SBPR15,

- c) production of the plasmid pSBPOCS making use of the SalI fragment of plasmid pSBPR15 1.9 kb in size,
 - d) integration of foreign genes into the pSBPOCS expression cassette,
 - 5 e) cloning of the expression cassette containing a DNA sequence for over-expression of foreign genes in plant seeds, into binary vectors
 - f) transfer of the expression cassette containing an
10 foreign gene under the control of the SBPR promoter into a plant cell.
12. Use of an expression cassette according to claims 1 to 7 for expression of homologous and heterologous genes in the seeds of transformed plants.
- 15 13. Use of an expression cassette according to claims 1 to 7 for expression of genes changing the storage capacity or the germination capability of seeds.
- 20 14. Use of the plasmids pBISBPR7, pBISBPR15, pSBPGUS, pPTVSBPRGUS and pSBPOCS or derivatives thereof for transformation of culture crops.
- 25 15. Use of the plasmids pBISBPR7, pBISBPR15, pSBPGUS, pPTVSBPRGUS and pSBPOCS or derivatives thereof for regulation of endogenous processes or for production of heterogenous products in culture crops.
- 30 16. Use of an expression cassette according to claims 1 to 7, wherein the transformed plants expressing new gene products or ones altered in the seeds are selected, genetically stable lines are bred and the gene products are extracted from the seeds of the transgenic plants.

17. Plant cell containing a plasmid according to claims 8 - 10.

18. Plant cell produced according to the method of claim 11.

5

19. Plant or plant tissues regenerated from a plant cell according to claims 12 or 13.

20. Plant according to claim 14, wherein it is a culture crop.

Abstract

The present invention relates to an expression cassette for expression of arbitrary genes in plant seed and the plasmids containing the expression cassette. The invention also includes the production of transgenic plant cells containing this expression cassette as well as the use of the plasmids in this expression cassette to produce transgenic plants. Fields of application of the invention are biotechnology, pharmacy and plant production.

The invention has the objective of placing seed-specific expression in transgenic plants on a basis suitable for a production of materials. It is based on the task of constructing an expression cassette with which a stable expression with a high expression rate of genes of the materials to be produced can be achieved in plant seeds.

The expression cassette according to the invention contains the following essential component parts:

- the promoter of the gene of the seed protein similar to the sucrose binding protein (SBP)
- if applicable, the DNA sequence of a signal peptide, preferably the SBP signal peptide
- a gene to be expressed
- 3' termination sequences

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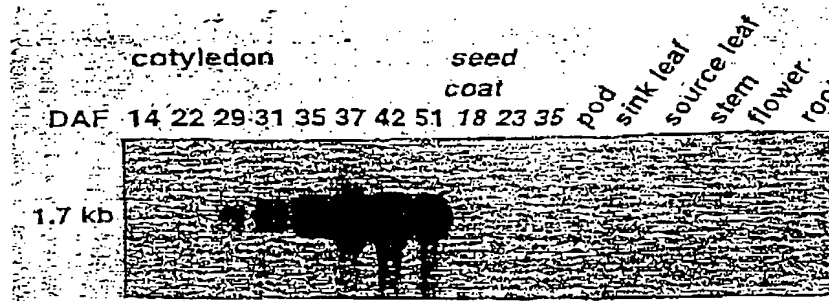
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~~Abb. 2~~

Fig. 2a



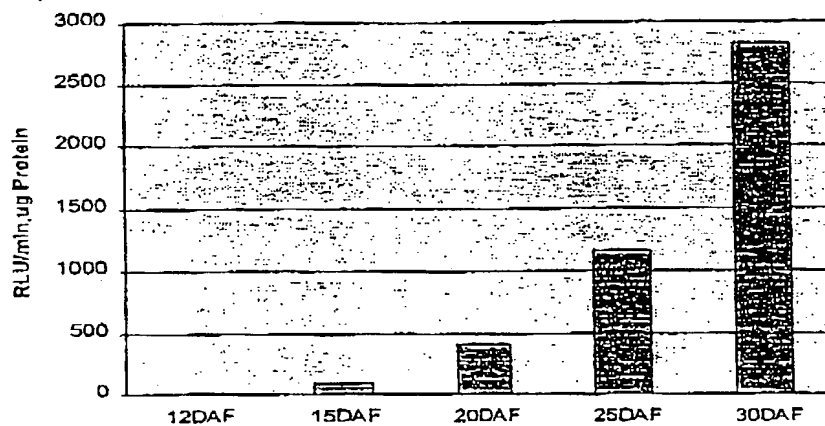
2a) Northern V.faba RNA gegen VfSBP20 Sonde,
Northern Blot of Vicia faba Against VFSBP20 probe

Fig. 2b



2b) Schnitt durch reife transgene (SBPRGUS) Tabaksamen
Cross-section of Ripe Transgenic (SBPRGUS) Tobacco Seed
GUS Gehalt in transgenen pSBPRGUS Tabak Linien
(n=15)
 β -glucuronidase Content in Transgenic pSBPRGUS Tobacco Line

Fig. 2c



2c)

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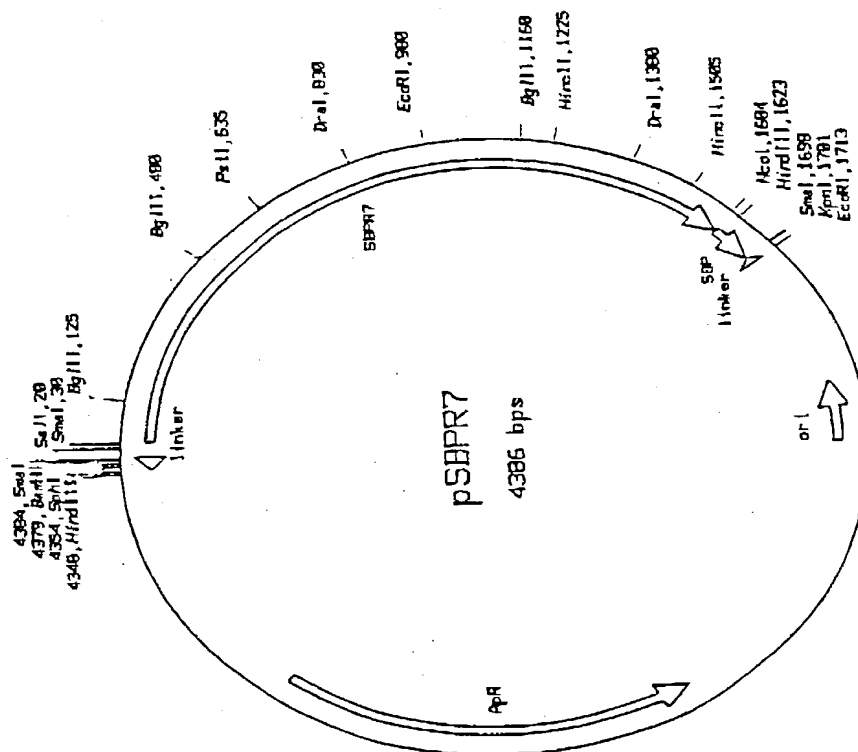
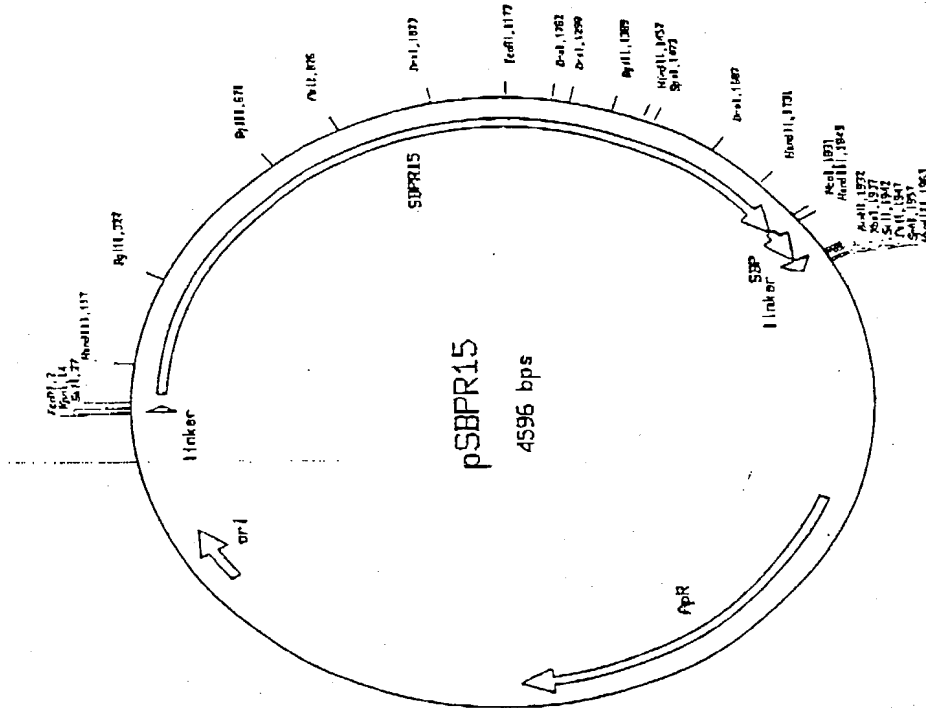


Abb. 3

Fig. 3

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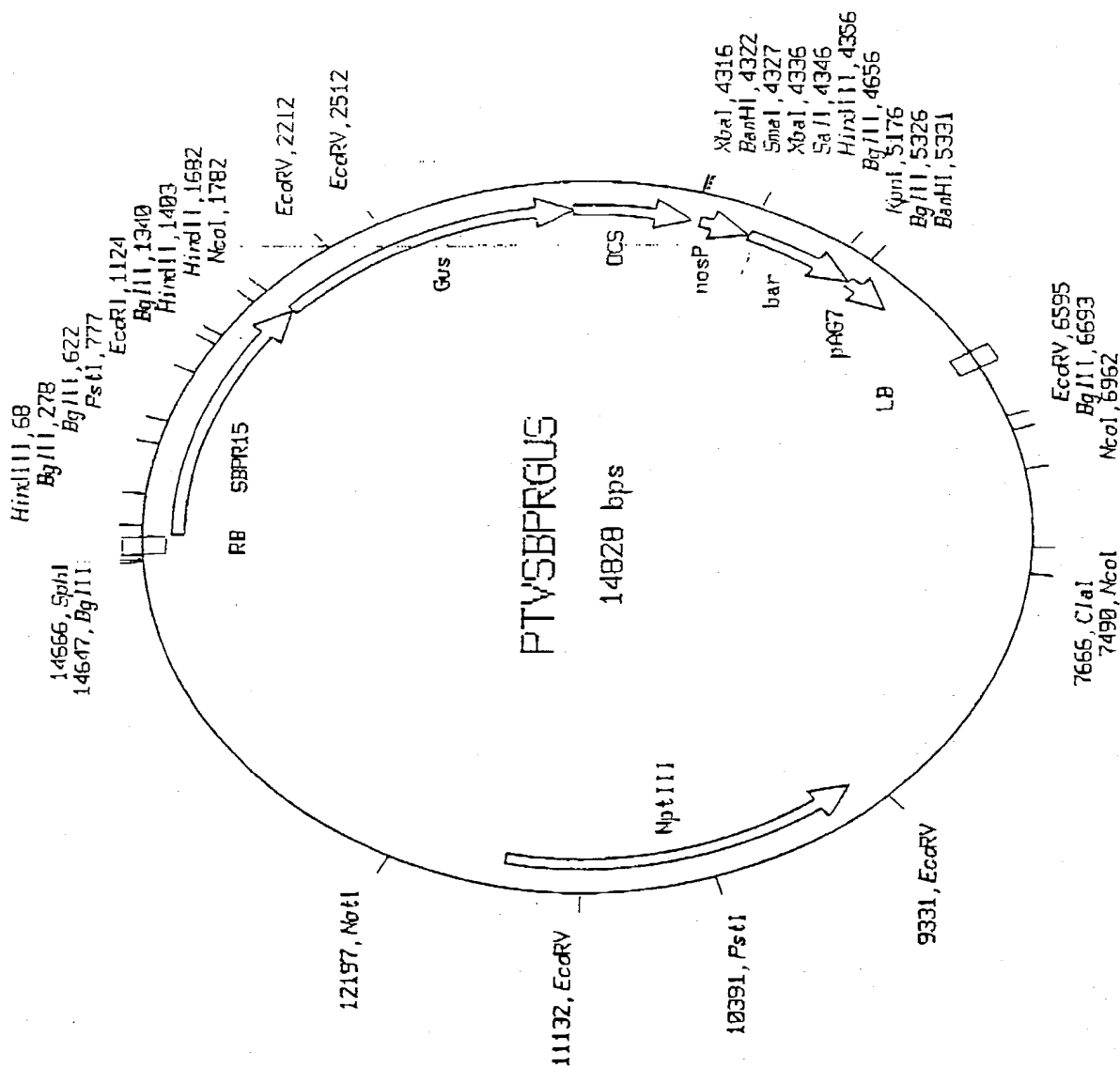


Abb. 4

Fig. 4

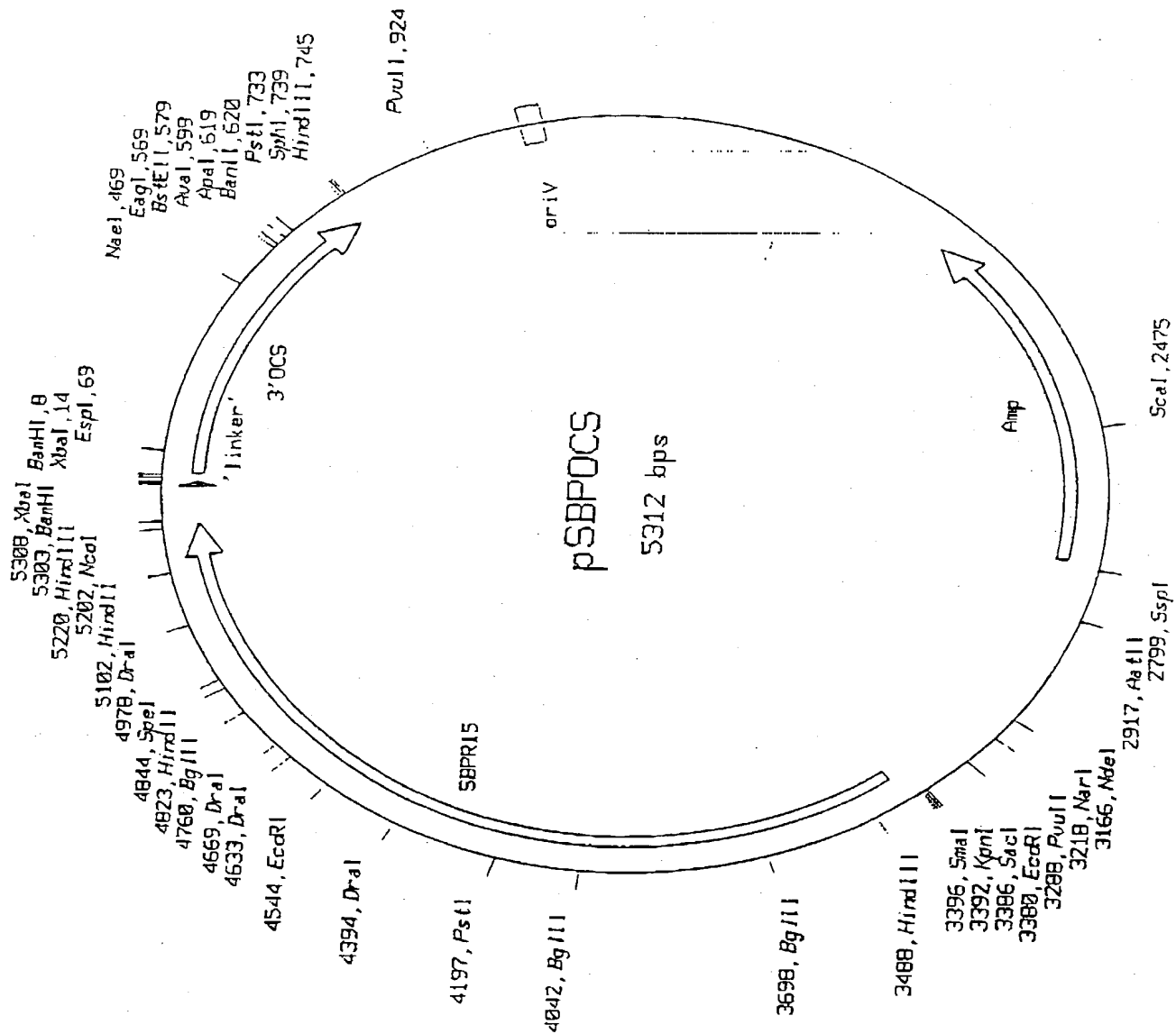


Abb. 5

Fig. 5

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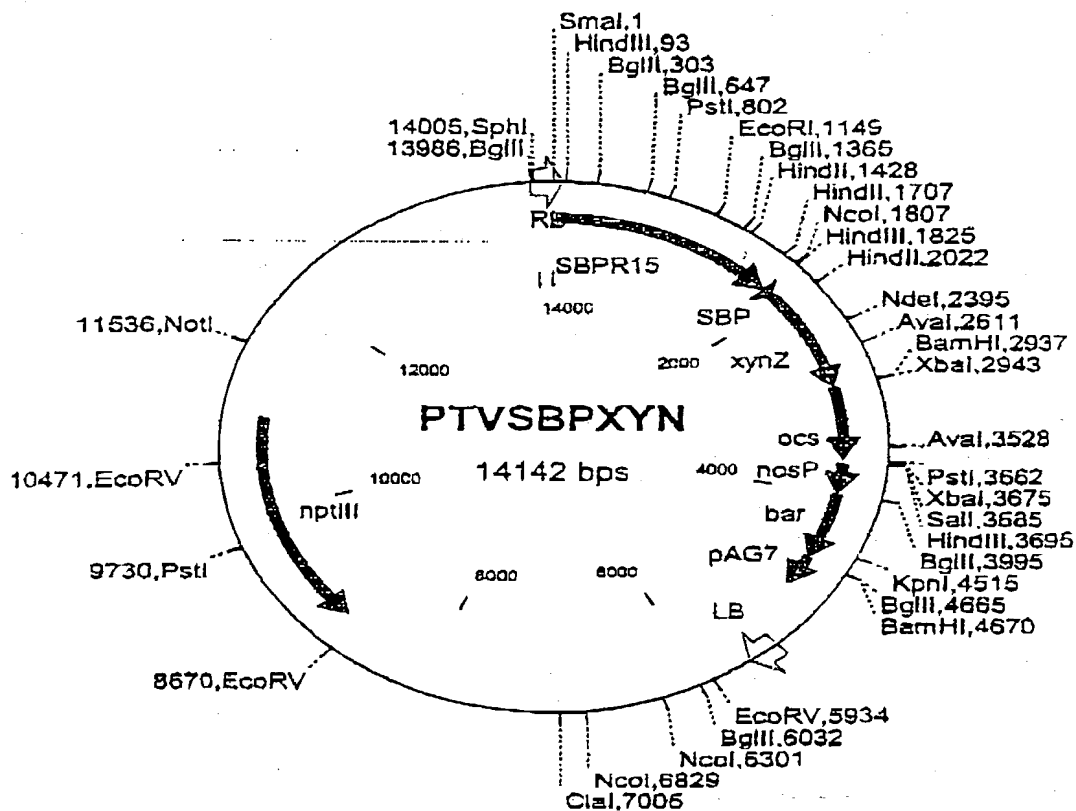


Abb. 6

Fig. 6

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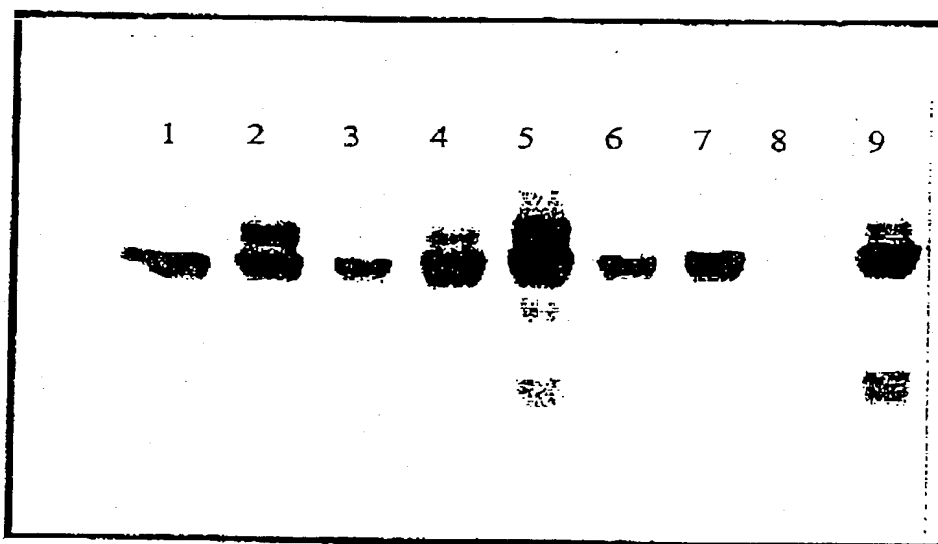


Abb. 7: Western Blot von Proteinextrakten aus reifen Samen mit gegen Xylanase Z gerichteten Antikörper:

1-7 unabhängige mit dem Plasmid PTVSBPXYN transformierte N. tabacum Linien; 8 Wildtyp; 9 positiv Kontrolle

Fig. 7: Western Blot of Protein Extract from Ripe Seed with Xylanase Z Directed Antibodies

Lanes 1-7 Independent with Plasmid PTVSBPXYN Transformed
N. Tabacum Line
Lane 8 Wild-type
Lane 9 Positive Control

Amended Claims - marked-up Copy

1. (amended) Promoter ~~A promoter for expression of arbitrary~~
5 ~~genes in plant seeds, wherein there exists the sequence of~~
~~Fig. 1a, which thus becomes the object of the claim.~~
2. (amended) Promoter ~~The promoter according to claim 1,~~
10 ~~wherein it mediates the expression in the cotyledons and in~~
~~the endosperm of seeds as a function of development.~~
3. (amended) Expression ~~An expression cassette for expression~~
15 ~~of arbitrary genes in the plant seed, containing comprising:~~
 - a promoter according to claim 1 ~~or 2,~~
 - a gene ~~to be capable of being expressed~~
 - 3' termination sequences.
4. (amended) Expression ~~The expression cassette according to~~
20 ~~claim 3, wherein it additionally contains the further~~
~~comprising a DNA sequence of a signal peptide, preferably~~
~~the SBP signal peptide.~~
5. (amended) Expression ~~The expression cassette according to~~
25 ~~claim 3, wherein further comprising a further second DNA~~
~~sequence is downstream to the a DNA region provided with a~~
~~transcriptionally regulatory sequence for a strong seed-~~
~~specific gene expression, the latter DNA region containing~~
~~the information for the formation and quantitative~~
30 ~~distribution of endogenous products or the expression of~~
~~heterologous products in culture crops.~~
6. (amended) Expression ~~The expression cassette according to~~
~~claims 3 to 5 claim 3, wherein arbitrary foreign genes are~~

integrated either as transcription or as translation fusions.

- 5 7. (amended) Expression ~~The expression cassette according to~~
~~claims 3 to 6~~ claim 4, wherein the signal peptide of the ~~is~~
coded by a SBP seed protein gene is used as a signal
peptide.
- 10 8. (amended) Expression cassette according to ~~claims 3 to 7,~~
wherein the gene ~~of the~~ is capable of coding for a sucrose
binding protein like gene ~~is used as the gene to be~~
expressed.
- 15 9. (amended) Expression ~~The expression cassette according to~~
~~claims 3 to 8~~ claim 3, wherein it is also used for co- and
multiple transformations.
- 20 10. (amended) Plasmids containing an expression cassette
~~according to claims 3 to 8~~ for expression of arbitrary
genes in the plant seed, comprising
 - a promoter according to claim 1
 - a gene capable of being expressed
 - 3' termination sequences.
- 25 11. (amended) Plasmid ~~pSBPROCS~~ The plasmid according to claim
10, wherein the plasmid is pSBPROCS comprising a DNA se-
quence about 5.3 kb in size, in which the DNA sequence
comprising a SalI promoter fragment of the regulatory
starter area about 1.9 kb in size including the signal
30 peptide and 5 triplets of the ~~a~~ SBP-homologous gene of
Vicia faba, restriction sites for cloning of foreign genes
and the ~~a~~ transcription terminator of the octopine synthase
gene are contained.

12. (amended) ~~Plasmid pPTVSBPRGUS~~ The plasmid according to claim 10, wherein the plasmid is pPTVSBPRGUS comprising a DNA sequence about 14.9 kb in size, ~~in which~~ comprising a phosphinothricin resistance gene about 1 kb in size, a SalI/NcoI promoter fragment of the regulatory starter area of the SBP-like gene of *Vicia faba* about 1.8 kb in size, the coding region of the β -glucuronidase about 2 kb in size and the transcription terminator of the octopine synthase gene ~~are contained~~.

13. (amended) Method for ~~the~~ an insertion of an expression cassette ~~according to claims 3 to 9~~ for expression of arbitrary genes in the plant seed, comprising a promoter according to claim 1, a gene capable of being expressed and 3' termination sequences with a DNA sequence for ~~strong seed-specific gene expression into a plant cell, comprising the following steps:~~

- a) ~~isolation of~~ isolating a clone VfSBP20, wherein the gene coding for the SBP seed protein occurring in the plant seed is selected from a cDNA Bank of cotyledons of *Vicia faba*,
- b) ~~isolation of~~ isolating a clone pSBPR15, wherein ~~the~~ a DNA sequence contained therein comprises the regulatory starter region of the SBP seed protein gene of *Vicia faba* and a sequence from a related legume hybridising with the DNA sequence of ~~the~~ SBPR15,
- c) ~~production of the~~ producing a plasmid pSBPOCS ~~making use of by~~ isolating and closing the SalI fragment of plasmid pSBPR15 1.9 kb in size,
- d) ~~integration of~~ integrating foreign genes into the pSBPOCS expression cassette,
- e) cloning of the expression cassette containing a DNA sequence for over-expression of foreign genes in plant seeds into binary vectors

f) ~~transfer of transferring~~ the expression cassette containing ~~an the~~ foreign gene under the control of the promoter ~~according to claims 1 or 2~~ into a plant cell for expression of arbitrary genes in plant seeds.

5

~~14. Use of an expression cassette according to claims 3 to 9 for expression of homologous and heterologous genes in the seeds of transformed plants.~~

10 ~~15. Use of an expression cassette according to claims 3 to 9 for expression of genes changing the storage capacity or the germination capability of seeds.~~

15 ~~16. Use of the plasmids pBISBPR7, pBISBPR15, pSBPGUS, pPTVSBPRGUS and pSBPOCS or derivatives thereof for transformation of culture crops.~~

20 ~~17. Use of the plasmids pBISBPR7, pBISBPR15, pSBPGUS, pPTVSBPRGUS and pSBPOCS or derivatives thereof for regulation of endogenous processes or for production of heterogenous products in culture crops.~~

25 ~~18. Use of an expression cassette according to claims 3 to 9, wherein the transformed plants expressing new gene products or such altered in the seeds are selected, genetically stable lines are bred and the gene products are extracted from the seeds of the transgenic plants.~~

30 19 (amended) Plant cell containing a plasmid according to claims 10 to 12 containing an expression cassette for expression of arbitrary genes in the plant seed, comprising a promoter according to claim 1, a gene capable of being expressed and 3' termination sequences.

20. (amended) Plant cell produced according to the
method of claim 13, wherein a plant cell is produced.

5 21. (amended) Plant or plant tissues regenerated from a plant
cell according to claims 14 or 15 based on an expression
cassette for expression of homologous and heterologous
genes in the seeds of transformed plants, comprising a
promoter according to claim 1, a gene capable of being
expressed, and 3' termination sequences.

10 22. (amended) Plant according to claim 1421, wherein it is a
culture crop.

15 23. Use of the DNA sequence of the SBP signal peptide in an expression cassette for expression
of arbitrary genes in plant seed.

24. (New) The expression cassette according to claim 4, further
comprising a DNA sequence of a SBP signal peptide.

Amended Claims - Clean Copy

1. (amended) A promoter for expression of arbitrary genes in
5 plant seeds.
2. (amended) The promoter according to claim 1, wherein it
mediates the expression in the cotyledons and in the
endosperm of seeds as a function of development.
- 10 3. (amended) An expression cassette for expression of
arbitrary genes in the plant seed, comprising:
 - a promoter according to claim 1,
 - a gene capable of being expressed
 - 15 • 3' termination sequences.
4. (amended) The expression cassette according to claim 3,
further comprising a DNA sequence of a signal peptide.
- 20 5. (amended) The expression cassette according to claim 3,
further comprising a second DNA sequence downstream to a
DNA region provided with a transcriptionally regulatory
sequence for a seed-specific gene expression, the DNA
region containing information for the formation and
25 quantitative distribution of endogenous products or
expression of heterologous products in culture crops.
6. (amended) The expression cassette according to claim 3,
wherein arbitrary foreign genes are integrated either as
transcription or as translation fusions.
- 30 7. (amended) The expression cassette according to claim 4,
wherein the signal peptide is coded by a SBP seed protein
gene.

8. (amended) Expression cassette according to, wherein the gene is capable of coding for a sucrose binding protein like gene.
- 5 9. (amended) The expression cassette according to claim 3, wherein it is also used for co- and multiple transformations.
- 10 10. (amended) Plasmids containing an expression cassette for expression of arbitrary genes in the plant seed, comprising
- a promoter according to claim 1
 - a gene capable of being expressed
 - 3' termination sequences.
- 15 11. (amended) The plasmid according to claim 10, wherein the plasmid is pSBPROCS comprising a DNA sequence about 5.3 kb in size, the DNA sequence comprising a SalI promoter fragment of the regulatory starter area about 1.9 kb in
- 20 size including the signal peptide and 5 triplets of a SBP-homologous gene of *Vicia faba*, restriction sites for cloning of foreign genes and a transcription terminator of the octopine synthase gene.
- 25 12. (amended) The plasmid according to claim 10, wherein the plasmid is pPTVSBPRGUS comprising a DNA sequence about 14.9 kb in size, comprising a phosphinothricin resistance gene about 1 kb in size, a SalI/NcoI promoter fragment of the regulatory starter area of the SBP-like gene of *Vicia faba*
- 30 about 1.8 kb in size, the coding region of the β -glucuronidase about 2 kb in size and the transcription terminator of the octopine synthase gene.

13. (amended) Method for an insertion of an expression cassette for expression of arbitrary genes in the plant seed, comprising a promoter according to claim 1, a gene capable of being expressed and 3' termination sequences with a DNA sequence for seed-specific gene expression into a plant cell, comprising the following steps:

- a) isolating a clone VfSBP20, wherein the gene coding for the SBP seed protein occurring in the plant seed is selected from a cDNA Bank of cotyledons of *Vicia faba*,
- b) isolating a clone pSBPR15, wherein a DNA sequence contained therein comprises the regulatory starter region of the SBP seed protein gene of *Vicia faba* and a sequence from a related legume hybridising with the DNA sequence of SBPR15,
- c) producing a plasmid pSBPOCS by isolating and closing the SallI fragment of plasmid pSBPR15 1.9 kb in size,
- d) integrating foreign genes into the pSBPOCS expression cassette,
- e) cloning of the expression cassette containing a DNA sequence for over-expression of foreign genes in plant seeds into binary vectors
- f) transferring the expression cassette containing the foreign gene under the control of the promoter for expression of arbitrary genes in plant seeds.

19. (amended) Plant cell containing a plasmid containing an expression cassette for expression of arbitrary genes in the plant seed, comprising a promoter according to claim 1, a gene capable of being expressed and 3' termination sequences.

20. (amended) The method of claim 13, wherein a plant cell is produced.

5 21. (amended) Plant or plant tissues regenerated from a plant cell based on an expression cassette for expression of homologous and heterologous genes in the seeds of transformed plants, comprising a promoter according to claim 1, a gene capable of being expressed, and 3' termination sequences.

10 22. (amended) Plant according to claim 21, wherein it is a culture crop.

15 24. (New) The expression cassette according to claim 4, further comprising a DNA sequence of a SBP signal peptide.

(12) NACH DEM VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES
PATENTWESENS (PCT) VERÖFFENTLICHTE INTERNATIONALE ANMELDUNG

BERICHTIGTE FASSUNG

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Internationales Büro



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- (48) Datum der Veröffentlichung dieser berichtigten Fassung: 26. Juli 2001

[Fortsetzung auf der nächsten Seite]

(54) Title: NOVEL EXPRESSION CASSETTE FOR EXPRESSING GENES IN PLANT SEED

(54) Bezeichnung: NEUE EXPRESSIONSKASSETTE ZUR EXPRESSION VON BELIEBIGEN GENEN IN PFLANZENSAMEN

(57) Abstract: The invention relates to an expression cassette for expressing genes in plant seed and to the plasmids containing said expression cassette. The invention includes the production of transgenic plant cells containing said expression cassette and the use of the plasmids in said expression cassette for producing transgenic plants. The invention can be applied in the field of biotechnology, pharmaceuticals and plant production. The aim of the invention is to provide a means for the seed-specific expression in transgenic plants in such a manner that it is suitable for the production of the desired substances. Another aim of the invention is to construct an expression cassette which allows stable expression of genes of substances to be produced in plant seed at a high expression rate. The inventive expression cassette comprises the following essential components: the promoter of the gene of the seed protein which is analogous to the sucrose binding protein (SBP), optionally the DNA sequence of a signal peptide, preferably that of the SBP signal peptide, a gene to be expressed, 3' termination sequences.

(57) Zusammenfassung: Die vorliegende Erfindung betrifft eine Expressionskassette zur Expression von beliebigen Genen in Pflanzensamen und die die Expressionskassette enthaltenden Plasmide. Die Erfindung schliesst die Herstellung transgener Pflanzenzellen, die diese Expressionskassette enthalten, sowie die Verwendung der Plasmide in dieser Expressionskassette zur Herstellung von transgenen Pflanzen mit ein. Anwendungsgebiete der Erfindung sind die Biotechnologie, die Pharmazie und die Pflanzenproduktion. Die Erfindung hat das Ziel, die samenspezifische Expression in transgenen Pflanzen auf eine für eine Stoffproduktion geeignete Basis zu stellen. Ihr liegt die Aufgabe zugrunde, eine Expressionskassette zu konstruieren, mit der eine stabile Expression mit hoher Expressionsrate von Genen der herzustellenden Stoffe in Pflanzensamen erreicht werden kann. Die erfindungsgemässe Expressionskassette enthält folgende wesentlichen Bestandteile: den Promotor des Gens des Saccharosebindeprotein (SBP)-ähnlichen Samenproteins; ggf. die DNA-Sequenz eines Signalpeptids, bevorzugt des SBP-Signalpeptids; ein zu exprimierendes Gen; 3'-Terminationssequenzen.

WO 00/26388 A3

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If each inventor understands English, the Declaration and Power of Attorney below is suitable for use when filing a regular patent application and also when entering the national stage, in the case of an International application designating the USA under the PCT.

COMBINED DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION			Attorney Docket No. 101195-48																								
<p>As a below named inventor, I hereby declare that: My residence, post office address and citizenship are as stated below next to my name, I believe I am the original, first and sole inventor (if only one name is listed below at 201) or an original, first and joint inventor (if plural names are listed below at 201-205) of the subject matter which is claimed and for which a patent is sought on the invention entitled</p> <p>New Expression Cassette for Expression of Arbitrary Genes in Plant Seeds</p> <p>the specification of which (check one)</p> <p><input type="checkbox"/> is attached hereto</p> <p><input type="checkbox"/> was filed on <u>27 October 1999</u></p> <p>under Serial Number <u>PCT/EP99/03432</u> and was amended on <u>28.12.2000</u> (if applicable).</p> <p>I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.</p> <p>I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, Section 1.56.</p> <p>I list below any prior foreign application(s) for patent or inventor's certificate in respect of which foreign priority benefits are claimed under 35 USC 119; and any prior foreign application(s) for patent or inventor's certificate in respect of which such foreign priority rights are not claimed and which has a filing date before that of any application in respect of which such foreign priority benefits are claimed:</p> <table border="1"><thead><tr><th>Application Number</th><th>Country</th><th>Filing Date (day, month, year)</th><th>Priority Claimed under 35 USC 119</th></tr></thead><tbody><tr><td>198 52 195.2</td><td>Germany</td><td>4 November 1998</td><td>YES: <input checked="" type="checkbox"/> NO: <input type="checkbox"/></td></tr><tr><td></td><td></td><td></td><td>YES: <input type="checkbox"/> NO: <input type="checkbox"/></td></tr><tr><td></td><td></td><td></td><td>YES: <input type="checkbox"/> NO: <input type="checkbox"/></td></tr></tbody></table> <p>I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below.</p> <table border="1"><thead><tr><th>Application No.</th><th>Filing Date</th></tr></thead><tbody><tr><td></td><td></td></tr><tr><td></td><td></td></tr><tr><td></td><td></td></tr></tbody></table>				Application Number	Country	Filing Date (day, month, year)	Priority Claimed under 35 USC 119	198 52 195.2	Germany	4 November 1998	YES: <input checked="" type="checkbox"/> NO: <input type="checkbox"/>				YES: <input type="checkbox"/> NO: <input type="checkbox"/>				YES: <input type="checkbox"/> NO: <input type="checkbox"/>	Application No.	Filing Date						
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Application No.	Filing Date																										

Combined Declaration and Power of Attorney
101195-48
Page 2

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

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	City of Residence	State or Foreign Country	Country of Citizenship
204	Post Office Address	City	State & ZIP/Country

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205	Family Name	First Given Name	Second Given Name
	City of Residence	State or Foreign Country	Country of Citizenship
	Post Office Address	City	State & ZIP/Country
<p>I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.</p>			
Signature of Inventor 201		<i>U. Kim</i>	Date <i>May, 28, 2001</i>
Signature of Inventor 202		<i>Heungs Wehn</i>	Date <i>May, 28, 2001</i>
Signature of Inventor 203			Date
Signature of Inventor 204			Date
Signature of Inventor 205			Date